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PRINCIPAL INVESTIGATOR: Wolfram Ruf, M.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

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Wolfram Ruf, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
LaJolla, California 92037

E-Mail: ruf@scripps.edu

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Tissue Factor (TF) is the cell surface receptor that activates coagulation by binding the serine protease coagulation factor VIIa (VIIa). The activation of the coagulation cascade leads to thrombin generation, fibrin formation and platelet activation which together may aide tumor growth and metastasis. TF is released from tumor cells by shedding or TF comes in contact with coagulation factors when tumor cells enter the blood stream, leading to a hypercoagulable state and its clinical manifestation of spontaneous thrombosis (Trousseau's Syndrome) that occurs in several types of cancer. This provides clear evidence that TF is a frequent marker of advanced cancer. This project proposes to elucidate mechanisms that underlie the seemingly paradoxical observation that TF which supports metastasis under certain conditions may actually delay tumor development and local spread in breast cancer. The basic hypothesis pursued here is that the complex signaling functions of the TF cytoplasmic domain contribute to this paradox. We hope to discover new avenues to specifically interrupt breast cancer progression by unraveling the role of TF cytoplasmic domain signaling in cancer cells.

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Introduction

Tissue Factor (TF) is the cell surface receptor that activates coagulation by binding the serine protease coagulation factor VIIa (VIIa) . The activation of the coagulation cascade leads to thrombin generation, fibrin formation and platelet activation which together may aide tumor growth and metastasis. TF is released from tumor cells by shedding or TF comes in contact with coagulation factors when tumor cells enter the blood stream, leading to a hypercoagulable state and its clinical manifestation of spontaneous thrombosis (Trousseau's Syndrome) that occurs in several types of cancer. This provides clear evidence that TF is a frequent marker of advanced cancer. This project proposes to elucidate mechanisms that underlie the seemingly paradoxical observation that TF which supports metastasis under certain conditions may actually delay tumor development and local spread in breast cancer. The basic hypothesis pursued here is that the complex signaling functions of the TF cytoplasmic domain contribute to this paradox. We hope to discover new avenues to specifically interrupt breast cancer progression by unraveling the role of TF cytoplasmic domain signaling in cancer cells.

Body

This application has proposed two specific aims. Aim 1 is to test in A7 melanoma cells whether TF suppresses tumor growth by TF cytoplasmic domain signaling and whether WW-domain proteins are involved in this suppression by binding to the phosphorylated TF cytoplasmic domain. The review committee was concerned by the use of a melanoma cell line to study mechanisms of breast cancer development. We have responded to this conceptual weakness in continuing our survey to identify a TF-negative breast cancer cells line. We were fortunate to identify two such clones that were established in the laboratory of our colleague Dr. B. Felding-Habermann here at Scripps. These breast cancer lines are known to produce tumors in immuno-deficient mice and metastasize aggressively. Theses biological properties suggest that these cell lines may be a suitable model to study mechanisms by which TF suppresses the development of breast cancer. We made the decision to continue our studies with these cell lines, instead of melanoma cells. Fig. 1 shows FACS analysis with anti-TF antibody of these cell lines, named BMS and BCM1. Both cell lines were transfected, as proposed originally, with an expression plasmid for human TF that can be regulated by tetracycline. Stable lines were successfully generated and addition of tetracycline suppressed TF expression in BMS/TF cells by 95% and in BCM1/TF cells by 84% (Fig. 1). Tumor growth experiments are currently ongoing in mice that do or do not receive

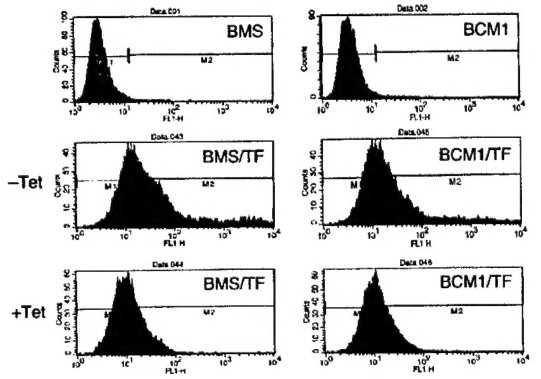

Fig. 1: TF expression on parental and TF transfected human breast cancer cell lines BCM1 and BMS. + Tet denotes that breast cancer cells were cultured in the presence of tetracycline to suppress TF expression.

tetracycline in their drinking water. In this experiment, tumor growth curves for both cell lines are established and tumors are isolated upon termination of the observation period to confirm TF expression levels in the tumors. This is important to exclude a survival advantage of TF negative escape mutants. The suppression of TF expression by tetracycline administration is further confirmed.

We have recently applied our phosphorylation-specific antibody against the TF cytoplasmic domain (1) to the analysis of human pathological specimens. In ocular, diabetic neovascularization we have made the discovery that TF phosphorylation is a hallmark of TF expressed under pathological conditions, but not in normal tissues (2). We also demonstrated in vitro that the TF cytoplasmic domain becomes phosphorylated when breast cancer cells are stimulated through PAR2 (3), established in vivo as the signaling receptor for TF (2). Thus, tools are available to directly test whether TF becomes phosphorylated in breast cancer cells. Therefore, specimens of the currently growing tumors are also embedded for frozen sections and the phosphorylation status of TF in our breast cancer model is determined. We are continuing the proposed studies with these newly established breast cancer models with the hope to have a model of highest relevance for breast cancer biology.

Aim 2 is to generate tumor prone animals by crossing hormone sensitive C3-TAg mice with TF cytoplasmic domain deleted ($TF^{\Delta CD/\Delta CD}$) mice. These crosses are progressing according to schedule. We have recently obtained male founders that lack the TF cytoplasmic domain and carry the C3-TAg transgene (Fig. 2). We have initiated crosses of these mice with ~ 20 female $TF^{\Delta CD/\Delta CD}$. Breeding of the control group was also initiated. To this end, C3-TAg wild-type mice are crossed with wild-type mice from our colony. We expect to establish a cohort of sufficient size for observation of tumor incidence and development in the upcoming year, as proposed.

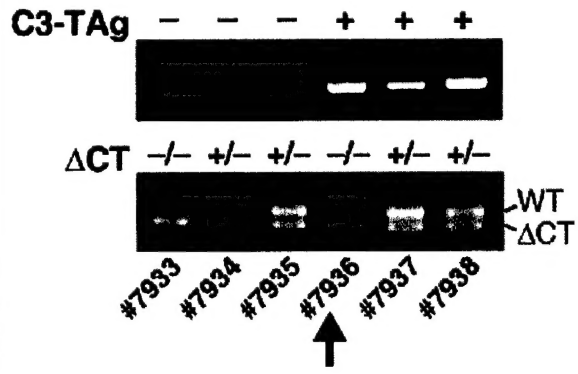

Fig. 2: Generation of C3-TAg/$TF^{\Delta CD/\Delta CD}$ mice. Genotyping of male offspring is shown. Male C3-TAg/$TF^{\Delta CD/\Delta CD}$ mice will be mated with $TF^{\Delta CD/\Delta CD}$ females to generate a cohort of breast cancer prone C3-TAg offspring.

Key Research Accomplishments

- Identified TF negative breast cancer cells
- Established breast cancer cell lines with tetracycline regulated TF expression
- Generated $TF^{\Delta CD/\Delta CD}$/C3-TAg founders and initiated breeding program

Reportable Outcomes

Our recent paper has established that the TF cytoplasmic domain is phosphorylated downstream of PAR2 signaling in breast cancer cells (Ahamed & Ruf. J.Biol.Chem. 279:23038, 2004)

Conclusions

We have made progress in identifying a novel cell model of potential utility to study the role of TF in breast cancer. We continue further studies in this model to conform with the recommendations of the review committee. Generation of breast cancer prone mouse strains progresses on schedule.

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Protease-activated Receptor 2-dependent Phosphorylation of the Tissue Factor Cytoplasmic Domain*

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Jasimuddin Ahamed and Wolfram Ruf‡

From the Scripps Research Institute, La Jolla, California 92037

Tissue factor (TF) is the physiological activator of the coagulation cascade that plays pathophysiological roles in metastasis, angiogenesis, and inflammation. Downstream in coagulation, thrombin is the central protease that signals through G protein-coupled, protease-activated receptors (PARs). However, the TF-VIIa-Xa complex upstream in coagulation also activates PAR1 and 2. Here, we address the question of whether signaling of the TF initiation complex is a relevant pathway that leads to TF cytoplasmic domain phosphorylation. In heterologous expression systems and primary endothelial cells, we demonstrate that the ternary TF-VIIa-Xa complex induces TF phosphorylation specifically by activating PAR2 but not through PAR1 signaling. In addition, TF cytoplasmic domain phosphorylation is induced only by TF-dependent signaling but not by other coagulation factors in endothelial cells. Phosphorylation of the Pro-directed kinase target site Ser^{258} is dependent on prior phosphorylation of Ser^{253} by protein kinase C (PKC) α. TF phosphorylation is somewhat delayed and coincides with sustained PKCα activation downstream of PAR2 but not PAR1 signaling. Phosphatidylcholine-dependent phospholipase C is the major pathway that leads to prolonged PKCα recruitment downstream of PAR2. Thus, PAR2 signaling specifically phosphorylates TF in a receptor cross-talk that distinguishes upstream from downstream coagulation protease signaling.

Cell signaling through G protein-coupled protease-activated receptors (PARs)[1] is closely linked to the activation of the coagulation protease cascade (1). Genetic evidence established an essential *in vivo* role of PAR signaling for platelet activation and the hemostatic response (2, 3). However, PAR signaling also has important nonhemostatic functions in development (4), inflammation (5), and tumor biology (6). All PARs, except for PAR2, are directly activated by thrombin, and PAR signaling is typically attributed to the action of thrombin *in vivo*. However, *in vitro* evidence indicates that thrombin receptors are also targets for other proteases at meaningful physiological concentrations (7–10). Cellular receptors can concentrate serine proteases at the cell surface for PAR cleavage (11). More importantly, protease co-factors, such as tissue factor (TF) and endothelial cell protein C receptor, couple the initiation of the pro- and anti-coagulant pathways to PAR signaling by retaining transiently the initial protease products Xa and activated protein C on the cell surface (9, 10).

Different PARs produce only partially redundant signaling responses in vascular cells. In the case of PAR1 and PAR2, the predominant endothelial cell expressed PARs, several immediate early gene induction events are overlapping, but only PAR1 signaling induces the mRNA for monocyte chemoattractant protein 1 (10). PAR signaling induces distinct cytoskeletal effects. PAR1 is a potent activator of the small GTPase Rho, which leads to the disruption of intercellular junctions and to increased permeability of the endothelium (12, 13). In contrast, PAR2 activation promotes cell motility by activation of the Rac/p21-activated kinase pathway and by forming a scaffolding complex containing β-arrestin and extracellular signal-regulated kinase (ERK). This complex is implicated in pseudopodia extension and chemotaxis (14, 15). PAR selectivity is corroborated by *in vivo* studies demonstrating that agonist peptides specific for PAR1 and PAR2 elicit selective responses in vascular tone (16, 17) and that developmental early embryonic lethality is specific for genetic deletion of PAR1 (4). Thus, protease signaling specificity is in part dependent on the signal transducing PAR, but additional complexity possibly arises from the co-factors/protease receptors that assist PAR cleavage.

In the initiation phase of coagulation, TF is involved in two signaling complexes: the TF-VIIa complex that activates PAR2 and the ternary TF-VIIa-Xa complex in which Xa cleaves PAR1 or PAR2 (8, 9, 18, 19). Because the ternary complex activates PAR1 and PAR2, it is an unresolved question whether immediate or delayed responses distinguish between TF-dependent signaling through PAR1 or PAR2. In this study we further characterize how the TF cytoplasmic domain becomes phosphorylated in endothelial cells. Although TF phosphorylation can be induced by phorbol 12-myristate 13-acetate (PMA) stimulation (20–22), to date no agonist pathway of potential physiological relevance has been identified that leads to TF cytoplasmic domain phosphorylation. Here, we show that PAR2 but not PAR1 signaling induces TF cytoplasmic domain phosphorylation. PMA-induced TF phosphorylation in endothelial cells was found to involve PKCα-mediated phosphorylation of Ser^{253} (22) and subsequent phosphorylation of Ser^{258} by a potential Pro-directed kinase (20–22). Whereas PKCα is activated downstream of PAR1 and PAR2 signaling, sustained membrane recruitment of PKCα is specific for PAR2 signaling and coincides with the onset of TF cytoplasmic domain phosphorylation. TF phosphorylation is a characteristic response of the initiation

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‡ To whom correspondence should be addressed: Dept. of Immunology, C204, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-2748; Fax: 858-784-8480; E-mail: ruf@scripps.edu.

[1] The abbreviations used are: PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; NAP, nematode anticoagulant protein; TF, tissue factor; ERK, extracellular signal-regulated kinase; TFCD, TF cytoplasmic domain; HUVEC, human umbilical vein endothelial cell(s); PI, phosphatidylinositol; PC, phosphatidylcholine; PLC, phospholipase C.

phase of coagulation and thus distinguishes PAR signaling in the upstream and downstream coagulation reaction.

EXPERIMENTAL PROCEDURES

Materials—VIIa, inactive Ser^{195} to Ala VIIa (iVIIa), factors IXa, X and Xa, thrombin, and PAR agonist peptides TFLLRNPNDK (PAR1) and SLIGRL (PAR2) were as described (8, 9, 23). A polyclonal goat antibody to the human TF extracellular domain and rabbit antibodies to the phosphorylated (αP-TFCD) or nonphosphorylated (α-TFCD) TF cytoplasmic domain were characterized in detail (22). Sequencing grade trypsin (Roche Applied Science), chromogenic substrate Spectrozyme FXa (American Diagnostics, Greenwich, CT), PAR2 antibody SAM11 and PKCα antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho- and non-phospho-ERK1/2 antibodies (Cell Signaling, Beverly, MA) were obtained commercially. Hirudin, conventional PKC inhibitors Gö6983 and Gö6976, PKCα inhibitor Safingol, and PKCβ inhibitor Hispidin were purchased from Calbiochem (San Diego, CA). Anti-PAR1 antibodies ATAP2 and WEDE15 were kindly provided by Dr. L. F. Brass (24). Recombinant nematode anticoagulant proteins 5 (NAP5) and c2 (NAPc2) (25, 26) were kindly provided by Dr. G. Vlasuk (Corvas International, San Diego, CA). Wild-type and kinase inactive PKCα (K368N) constructs were gifts from Dr. Alex Toker (Boston, MA).

Cell Culture and Transient Transfection—Chinese hamster ovary (CHO) cells expressing full-length TF (CHO/TF cells) (27) were transiently transfected by electroporation. Cells (2×10^7) in 200 μl of Dulbecco's modified Eagle's medium, 2% fetal bovine serum, 1 mM HEPES combined with 20 μg of PAR1 or PAR2 cDNA constructs received a single pulse (250 V, 500 microfarads) in a gene pulser (Bio-Rad). The experiments were performed 48 h after transfection.

Adenoviral Vectors and Transduction of Endothelial Cells—Ad5 serotype vectors for full-length and C245S-mutated TF were described in detail (22). Ser^{253} to Ala TF, generated by PCR mutagenesis, and PAR1 and PAR2 cDNA were similarly subcloned into adenoviral vectors. The virus was plaque-purified twice, expanded for large scale production, and purified on CsCl gradient. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) or Cascade Biologics (Portland, OR) and cultured in EGM medium containing 2–5% fetal bovine serum, 1 mg/ml hydrocortisone, 10 μg/ml human epidermal growth factor, and 3 mg/ml bovine brain extract. HUVECs were transduced for 4–6 h, and the experiments were performed 48 h later. Typically, the cells were transferred to serum-free medium for 2–4 h with the fresh medium, before the agonists were added. To monitor the levels of Xa, an aliquot of culture supernatant was quenched in buffer containing EDTA, and the Xa concentrations were determined by hydrolysis of the chromogenic substrate Spectrozyme FXa, based on calibration curves of purified Xa.

Western Blotting and Fluorescence-activated Cell Sorter Analysis—From cells treated with inhibitors and agonists, TF was immunoprecipitated in the case of CHO cells and PAR1-deficient fibroblasts. MDA-MB-231 breast cancer cells or endothelial cells were directly lysed in reducing sample buffer for Western blot analysis with phosphorylation (αP-TFCD) or pan-specific (α-TFCD) antibody to the TF-cytoplasmic domain (22). To assess surface expression of TF, PAR1, or PAR2, the cells were harvested with EDTA and resuspended in serum-free medium, 2 mM $CaCl_2$. To determine PAR internalization, the cells were stimulated with ternary TF-VIIa-Xa (10 nM iVIIa, 20 nM Xa, 100 nM NAPc2) complex or PAR agonist peptides for 30 min at 37 °C. Residual expression was detected by staining on ice for PAR1 (WEDE-15) or PAR2 (SAM11), followed by fluorescein isothiocyanate-labeled secondary antibody and flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

Translocation of PKCα from Cytosol to Membrane—Transduced, serum-starved (4 h) HUVECs were stimulated with ternary complex (iVIIa/Xa/NAPc2, 10/20/100 nM) or PMA (20 ng/ml) for 1 and 10 min and washed with ice-cold phosphate-buffered saline, 1 mM orthovanadate. The cells were harvested by scraping in 20 mM Tris-HCl, pH 7.6, 1 mM EGTA, 2 mM EDTA, aprotinin (5 μg/ml), leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and sonicated twice (10 s pulse; Heat System, Ultrasonic Inc.). Cell debris was removed by centrifugation at 500 × *g* for 2 min, and the membrane and cytosolic fractions were separated by centrifugation of the supernatant at 100,000 × *g* for 1 h at 4 °C (28). The membrane fractions were resuspended in the same buffer, 1% Triton X-100, and the samples were separated by SDS-PAGE using equal protein loading for Western blotting with anti-PKCα antibody. The presented data are representative of experiments repeated at least three times. The quantitations are shown as the means and standard deviations.

RESULTS

TF Cytoplasmic Domain Phosphorylation Is Dependent on PAR2 Signaling in Heterologous Expression Systems—PAR signaling may activate PKC, which in PMA-stimulated cells triggers TF cytoplasmic domain phosphorylation (20–22). In TF transfected CHO (CHO/TF) cells, the TF cytoplasmic domain was phosphorylated in response to PMA stimulation, demonstrating expression of relevant kinases that phosphorylate TF. However, neither PAR1 nor PAR2 agonists induced TF cytoplasmic domain phosphorylation (Fig. 1*A*). CHO/TF cells express endogenous PAR1, but not PAR2 (9). To address the role of PAR2, CHO/TF cells were transfected to express human PAR2 (CHO/TF/PAR2) (9) or uncleavable PAR2 in which the P1 Arg residue was changed to Gln (CHO/TF/PAR2/P1). PAR2 agonist peptide induced TF cytoplasmic domain phosphorylation in both cell lines (Fig. 1*B*). To induce TF-dependent signaling, we used NAPc2 as an experimental tool to lock in the ternary TF-VIIa-Xa complex. In the NAPc2 stabilized TF-VIIa-Xa complex, VIIa is inhibited, but Xa is catalytically active and signaling competent, which mimics the characteristics of signaling in TF-initiated coagulation, where Xa is generated on cells exposed to VIIa and X (9). Unlike PAR2 agonist stimulation, the NAPc2 stabilized TF-VIIa-Xa signaling complex induced TF cytoplasmic domain phosphorylation only in cells that expressed cleavage-sensitive PAR2. Thus, cleavage of PAR2 by the ternary TF-VIIa-Xa complex induces TF cytoplasmic domain phosphorylation, and simply forming the ternary complex is not sufficient to induce TF cytoplasmic domain phosphorylation in PAR2-expressing cells.

To exclude the possibility that the lack of phosphorylation in response to PAR1 agonist stimulation in CHO/TF cells was due to low expression levels of hamster PAR1, CHO/TF cells were transiently transfected with either human PAR1 or human PAR2. Receptor expression was confirmed by flow cytometry (Fig. 1*C*). Expression of PAR1 or PAR2 resulted in similar PAR agonist or ternary complex-mediated ERK phosphorylation (Fig. 1*D*), as well as in comparable degrees of agonist-induced PAR1 or PAR2 internalization (Fig. 1*E*). These data are consistent with similar efficiency of PAR1 or PAR2 activation by ternary complex signaling. However, only PAR2 expression resulted in ternary complex-induced TF cytoplasmic domain phosphorylation (Fig. 1*D*). Thus, despite activating PAR1 as well as PAR2, ternary complex signaling caused TF cytoplasmic domain phosphorylation specifically through PAR2 activation.

PAR2 Signaling Is Sufficient to Support TF Cytoplasmic Domain Phosphorylation—Because endogenous PAR1 was co-expressed in PAR2-transfected CHO cells, it remained unclear whether PAR1 is required, albeit not sufficient to trigger TF phosphorylation. To address this question, fibroblasts from PAR1-deficient mice that also lack other PARs (9, 19) were used. PMA-stimulated TF cytoplasmic domain phosphorylation is negatively regulated by palmitoylation of the cytoplasmic Cys^{245} residue in endothelial cells (22), and mutation of Cys^{245} greatly facilitates the detection of TF cytoplasmic domain phosphorylation. PAR-1-deficient fibroblasts were co-transduced with adenovirus to express human Cys^{245} to Ser mutated TF together with PAR2 or PAR1, achieving expression levels shown in Fig. 2*A*. The cells were stimulated with PAR1 or PAR2 agonist peptides, and with thrombin, trypsin, or ternary complex as relevant proteases that activate PARs. No TF phosphorylation was observed in PAR1-expressing cells, but TF cytoplasmic domain phosphorylation was observed in PAR2-transduced cells stimulated with appropriate PAR2 agonists (Fig. 2*B*). These data demonstrate that endogenous PAR1 is not required in the signaling pathway that phosphorylates TF. Note the more quickly migrating form of TF in the loading

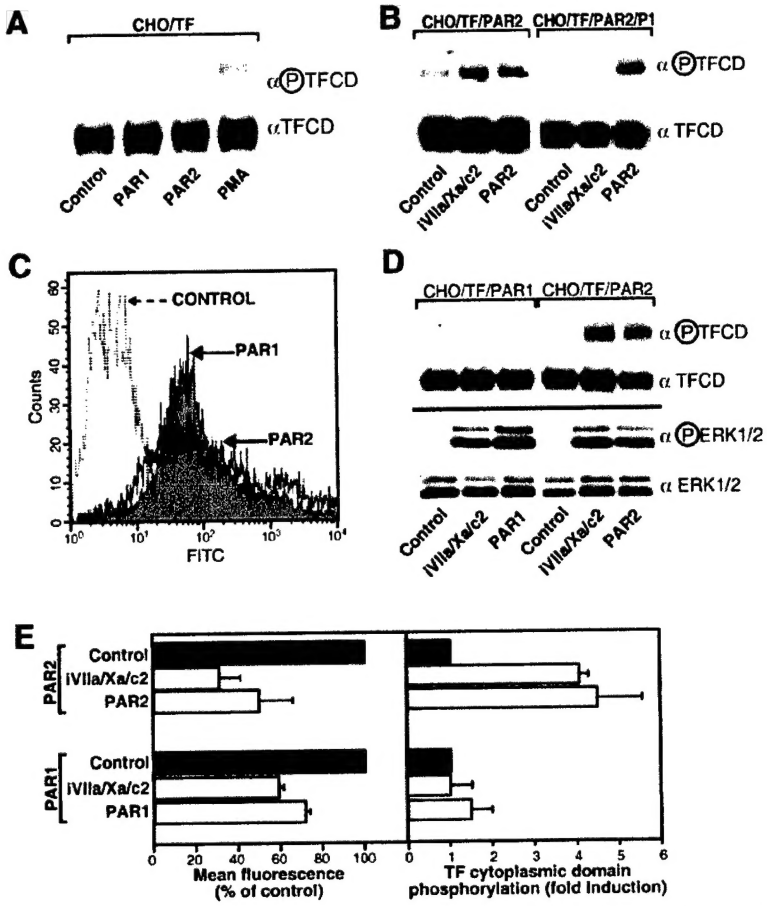

FIG. 1. **TF cytoplasmic domain phosphorylation is mediated by PAR2 signaling in heterologous expression systems.** *A*, serum-starved CHO/TF cells were stimulated with 10 μM PAR1 agonist, 100 μM PAR2 agonist, or PMA (20 ng/ml) for 1 h. Immunoprecipitated TF was detected by Western blotting with anti-phosphorylated TF cytoplasmic domain antibody (αP-TFCD) or pan-specific anti-TF cytoplasmic domain antibody (αTFCD). *B*, CHO/TF cells stably expressing a noncleavable mutant of PAR2 (CHO/TF/PAR2/P1) or wild-type PAR2 (CHO/TF/PAR2) were stimulated with PAR2 agonist peptide or NAPc2-stabilized ternary TF-VIIa-Xa complex (iVIIa/Xa/NAPc2 10/20/100 nM) for 1 h, followed by Western blotting for TF phosphorylation. Loading was controlled by reprobing with pan-specific anti-TF cytoplasmic domain antibody. *C*, transiently transfected CHO/TF cells were analyzed for human PAR expression by flow cytometry using WEDE15 or SAM11 monoclonal antibodies to PAR1 or PAR2, respectively. Controls were stained with isotype matched primary antibody. *D*, CHO/TF cells transiently expressing human PAR1 or PAR2 were stimulated with ternary complex or PAR agonists for 10 min for ERK1/2 phosphorylation or for 1 h for TF cytoplasmic domain phosphorylation. The loading control in the *top panel* by reprobing and the anti-ERK1/2 blot were performed on separate transfers. *E*, *left panel*, quantitation of receptor internalization after 30 min (37 °C) stimulation by ternary complex or PAR agonist of CHO/TF cells transiently transfected with human PAR1 or PAR2 (means and standard deviations, $n = 3$). *Right panel*, the increase in TF cytoplasmic domain phosphorylation, as shown in *D*, was quantified by laser densitometry (means and standard deviations, $n = 3$). FITC, fluorescein isothiocyanate.

control that was performed by reprobing with pan-specific anti-TF cytoplasmic domain antibody. We had previously found in PMA-stimulated cells that certain *N*-linked carbohydrate isoforms of TF cannot be phosphorylated (22). The lack of phosphorylation of the more quickly migrating forms of TF in this and other experiments shown below indicates that Golgi-dependent carbohydrate modification, and thus presumably a certain subcellular protein trafficking route, is also important for phosphorylation of the TF cytoplasmic domain in response to PAR2 activation.

TF Cytoplasmic Domain Phosphorylation of Endogenously Expressed TF—To provide evidence that endogenously expressed TF can be phosphorylated downstream of PAR2 signaling, we studied MDA-MB-231 cells. Under our culture conditions, this breast cancer cell line expressed fairly low levels of PAR2 but high levels of PAR1 and TF (Fig. 2*C*). MDA-MB-231 cells were stimulated by directly adding PAR1 or PAR2 agonist peptides to the culture supernatant. Sustained TF cytoplasmic domain phosphorylation was observed in cell stimulated with PAR2 but not in cells stimulated with PAR1 agonist peptide (Fig. 2*D*). The robust phosphorylation in response to PAR2 agonist peptide is remarkable, considering the fairly low levels of cell surface-expressed PAR2 relative to PAR1. Thus, in cells that express endogenous TF, PAR1, and PAR2, TF cytoplasmic domain phosphorylation occurs specifically downstream of PAR2 signaling.

TF Cytoplasmic Domain Phosphorylation Is Specific for Upstream Coagulation Signaling in Primary Cells—The relative importance of PAR1 and PAR2 signaling in TF cytoplasmic domain phosphorylation was analyzed in primary HUVECs transduced with adenovirus to express TF and variable levels of PAR1 and PAR2. HUVECs express endogenous PAR1 (Fig. 3*A*, *UT*) and very little PAR2 (data not shown). HUVECs were adenovirus transduced to achieve comparable expression levels of PAR1 or PAR2. PAR activation was confirmed by measuring internalization by flow cytometry and stimulation with the NAPc2 stabilized ternary complex similarly induced internalization of both receptors (Fig. 3*B*). With both PAR1 and PAR2, internalization by TF-dependent signaling was somewhat less efficient when compared with the cleavage by thrombin and trypsin, respectively.

Consistent with data in the CHO cell model, activation of

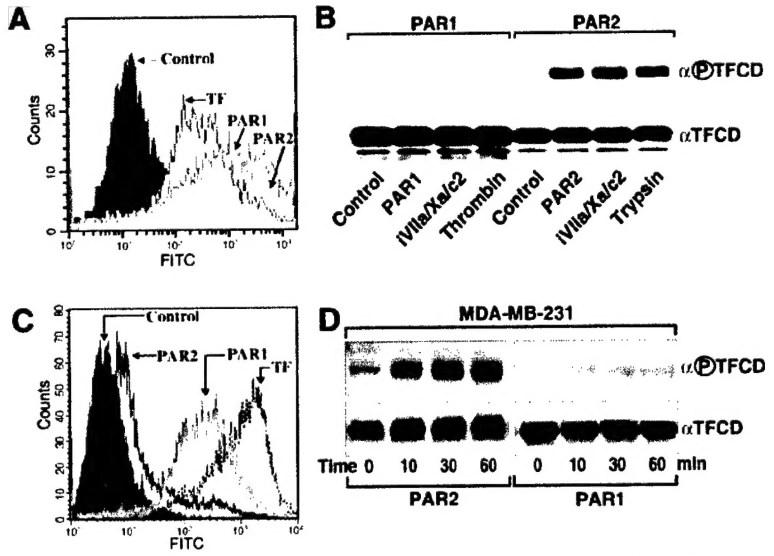

FIG. 2. **PAR2 dependence of TF phosphorylation in fibroblasts and breast cancer cells.** *A* and *B*, PAR1 signaling does not contribute to PAR2-mediated TF cytoplasmic domain phosphorylation. *A*, PAR-deficient M611 fibroblasts from PAR1$^{-/-}$ mice were co-transduced with Cys^{245} to Ser mutated TF and PAR1 or PAR2. Cell surface expression of TF, PAR1, and PAR2 was determined by flow cytometry, as in Fig. 1*C*. *B*, M611 cells co-expressing Cys^{245} to Ser mutated TF with PAR1 or PAR2 were stimulated with the indicated agonists and analyzed for TF cytoplasmic domain phosphorylation. Reprobing of the same blot with anti-TFCD was used to control for loading. *C* and *D*, phosphorylation of endogenously expressed TF in MDA-MB-231 human breast cancer cells downstream of PAR2 signaling. *C*, expression of TF, PAR1, and PAR2 in EDTA-lifted MAD-MB-231 cells was analyzed by flow cytometry. *D*, MDA-MB-231 cells were passaged by EDTA detachment and grown for 24 h. Cells were stimulated by addition to the medium of PAR1 or PAR2 agonist peptides for the indicated times. TF phosphorylation was determined by Western blotting with phosphorylation specific antibody and loading control by reprobing with pan-specific anti-TF cytoplasmic domain antibody. FITC, fluorescein isothiocyanate.

endogenous PAR1 (not shown) or overexpressed PAR1 did not induce TF phosphorylation (Fig. 3*C*). In contrast, introducing PAR2 with TF resulted in TF cytoplasmic domain phosphorylation after 10 min of stimulation with ternary complex, PAR2 agonist, or trypsin. Co-expression of PAR2 with Cys^{245} to Ser mutated TF at similar levels as wild-type TF resulted in significantly enhanced TF cytoplasmic domain phosphorylation after 10 min of stimulation (Fig. 3*C*) and PAR1 stimulation was also not sufficient to induce phosphorylation of this mutant (data not shown). The differences in TF phosphorylation between wild-type and mutant TF were quantified by densitometry, using normalization for slight differences in expression levels. TF was phosphorylated ~7-fold more efficiently when thioester modification of Cys^{245} was prevented by mutagenesis (n = 3).

The specificity of TF cytoplasmic domain phosphorylation in response to other coagulation proteases was analyzed in HUVECs expressing PAR2 and Cys^{245} to Ser mutated TF. The cells were stimulated for 1 h to increase the sensitivity for detecting inefficient responses, but high concentrations of factor IXa and activated protein C as well as thrombin did not induce TF phosphorylation (Fig. 3*D*). These are important data, because thrombin-cleaved PAR1 has been shown to cross-activate PAR2 (24). Whereas the NAPc2 stabilized TF-VIIa-Xa ternary complex triggered TF cytoplasmic domain phosphorylation with efficiency similar to that of a direct PAR2 agonist, signaling by VIIa (50 nM) alone produced less efficient TF phosphorylation in HUVEC monolayers. In contrast, stimulation with VIIa and X, which mimics activation of coagulation, efficiently induced TF cytoplasmic domain phosphorylation. In these experiments, Xa activity was blocked by adding a Xa inhibitor (NAP5) before free Xa reached levels that could activate PAR2.

The efficiency of ternary complex signaling is further illustrated in the *middle panel* of Fig. 3*D* with cells stimulated for only 10 min. The reaction with VIIa and X generated <10 nM Xa in this time period but lead to more efficient TF phosphorylation in comparison with free Xa at concentrations as high as 50 nM. In the *right panel*, the cells were stimulated with VIIa and X in the presence of the potent Xa inhibitor NAP5. Blocking Xa activity prevented TF cytoplasmic domain phosphorylation, confirming our previous conclusions (9) that in the ternary TF-VIIa-Xa coagulation initiation complex signaling is induced by Xa and not VIIa. Taken together, these experiments provide evidence that phosphorylation of the TF cytoplasmic domain occurs as a result of the activation of a highly specific signaling pathway following the initiation of coagulation.

PAR2 Signaling Induces Sustained PKCα Activation—The phosphorylation-specific antibody to TF recognizes the conformational change induced by phosphorylation of the cytoplasmic Ser^{258} residue that probably is the target for a Pro-directed kinase (20–22). However, PKCα-dependent phosphorylation of Ser^{253} appears to be required for PMA-induced Ser^{258} phosphorylation in HUVEC (22). Mutation of either the PKC phosphorylation site Ser^{253} or the PKC consensus recognition residues Lys^{255} abolished TF phosphorylation induced by PAR2-dependent ternary complex signaling (Fig. 4*A*). TF cytoplasmic domain phosphorylation was also blocked by inhibitors for conventional PKCs (α, β, and γ), Gö6976, and Gö6983, as well the PKCα-selective inhibitor Safingol but not the PKCβ-inhibitor Hispidin (Fig. 4*B*). To further demonstrate PKC specificity, we overexpressed wild-type PKCα or kinase-inactive, Lys^{368} to Asn mutated PKCα constructs. Ternary complex-induced TF cytoplasmic domain phosphorylation was significantly inhibited in cells expressing dominant negative, inactive PKCα, as compared with vector control or wild-type PKCα transfected cells (Fig. 4*C*). Thus, PKCα is important for TF cytoplasmic domain phosphorylation downstream of PAR2, presumably by targeting the PKC phosphorylation site at Ser^{253}.

Thrombin-mediated PAR1 signaling activates PKCα in endothelial cells to increase permeability (29, 30), but PAR2-mediated PKCα activation is poorly characterized. TF cytoplas-

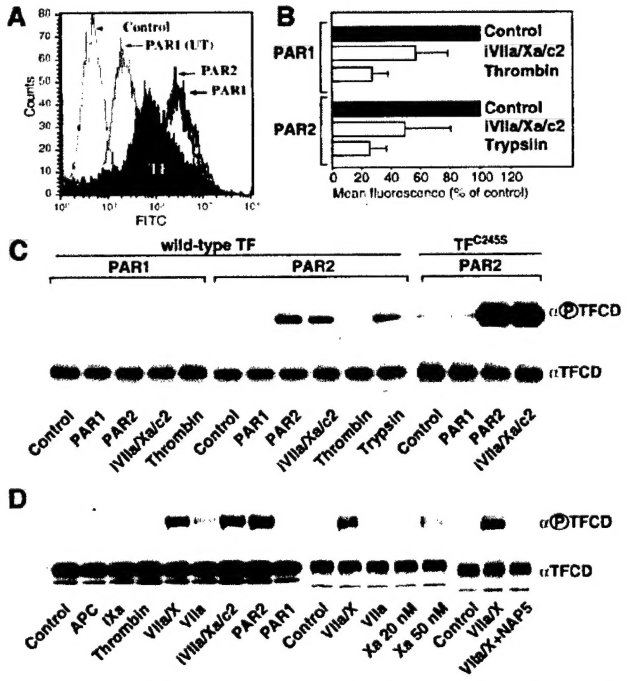

FIG. 3. **Protease specificity of PAR2-dependent TF cytoplasmic domain phosphorylation in endothelial cells.** *A*, co-expression of TF and PAR1 or PAR2 in HUVECs. Surface expression of TF, PAR1, and PAR2 was detected by flow cytometry 48 h after adenoviral transduction. Isotype-matched control staining was performed on untransduced cells. *PAR1 (UT)* depicts PAR1 expression levels without adenoviral transduction, and *PAR1* and *PAR2* are typical expression levels obtained at the chosen virus dose. *B*, PAR1 and PAR2 internalization in HUVECs stimulated for 30 min at 37 °C with the indicated agonists was determined by flow cytometry (means and standard deviation of residual mean fluorescence, $n = 3$). C, HUVECs co-transduced as in *A* to express either wild-type TF or Cys^{245} to Ser mutated TF and PAR1 or PAR2 were stimulated for 10 min with the indicated agonists and analyzed for TF phosphorylation by Western blotting. *D, left panel*, TF cytoplasmic domain phosphorylation is specific for upstream coagulation protease signaling. HUVECs transduced with Cys^{245}Ser TF and PAR2 were changed to serum-free medium and equilibrated for 2 h, followed by the addition of the indicated agonists for 1 h. 1 μM NAP5 was added after 10 min to prevent free Xa signaling in cells stimulated with VIIa/X. *Middle* and *right panels*, cells expressing Cys^{245}Ser TF and PAR2 were stimulated with factor X (100 nM) and VIIa (10 nM) or free Xa for 10 min. NAP5 was added at 1 μM just prior to the addition of VIIa and X to quench Xa activity immediately. TF cytoplasmic domain phosphorylation was determined by Western blotting. Controls for TF expression were performed on separate Western blots. For all panels, PAR1 agonist (10 μM), PAR2 agonist (100 μM), ternary complex iVIIa/Xa/NAPc2 (10/20/100 nM), thrombin (20 nM), activated protein C (10 nM), trypsin, VIIa alone, and IXa (50 nM) were used. For adenoviral co-transduction, wild-type and Cys^{245} to Ser mutated TF (200 virus particles/cell) and PAR1 and PAR2 (600 virus particles/cell) were used.

mic domain phosphorylation induced by PAR2 agonist or ternary complex signaling occurs with somewhat delayed kinetics, being undetectable 1 min and reaching maximal levels 5–10 min after stimulation (Fig. 5*A*). Translocation of conventional PKCs from the cytosol to the membrane is required for their kinase activity (31). To analyze the relation of TF cytoplasmic domain phosphorylation and PKCα activation, we determined the membrane translocation of PKCα at the 1- and 10-min time points. Fig. 5*B* shows that the ternary complex stimulated rapid (1 min) PKCα translocation from the cytosolic to the membrane fraction, but translocation was only transient and not detectable after (10 min) of stimulation of PAR1 expressing cells. In contrast, the cells expressing PAR2 showed not only rapid (1 min) redistribution of PKCα to the membrane but also sustained recruitment (10 min) after ternary complex signaling. Fig. 5*C* summarizes densitometric quantitation of repeat experiments, documenting that the prolonged PAR2-dependent translocation of PKCα was highly reproducible. PMA stimulation triggers TF cytoplasmic domain phosphorylation even in the absence of PAR2. PMA stimulation of either untransduced (not shown) or PAR2-transduced endothelial cells also resulted in prolonged PKCα translocation (Fig. 5*B*). Taken together, these experiments provide evidence that the duration of PKCα activity at the membrane is an important determinant for TF cytoplasmic domain phosphorylation.

A critical question, therefore, is how PAR2 signaling differs from PAR1 activation to induce sustained PKCα translocation. G protein-coupled receptor activation induces phosphatidylinositol (PI) breakdown mediated by PI-specific phospholipase C (PLC) isoforms to generate diacylglycerol required for PKC membrane recruitment. However, diacylglycerol is also generated by phosphatidylcholine-specific PLC (PC-PLC), which is known to promote prolonged membrane recruitment of PKCs (32). To analyze the role of PI- and PC-PLC in PKCα activation downstream of PAR2, the effect of PLC isoform specific inhibitors on TF cytoplasmic domain phosphorylation was analyzed. Whereas the PI-PLC specific inhibitor U73122 was without effect, the PC-PLC-specific inhibitor tricyclodecanoyl xanthogenate (D609) dose-dependently inhibited ternary complex- or PAR2 agonist-induced TF cytoplasmic domain phosphorylation (Fig. 5*D* and data not shown). To further provide evidence that D609 inhibits the pathway downstream of PAR2 leading to PKC activation, we tested the effect of D609 on TF phosphorylation by PMA, which directly activates PKC. Both D609 and U73122 only marginally reduced PMA-induced TF phosphorylation, demonstrating that pathways downstream of PKC are not influenced by D609.

The effect of PLC isoform-specific inhibitors on PKCα translocation was analyzed to clarify PAR2-mediated PKCα activation. In PAR2 expressing cells, the PC-PLC-specific inhibitor D609 significantly inhibited both initial and sustained PKCα membrane recruitment in response to TF ternary complex signaling. U73122 produced little to no effect on PKCα translocation in PAR2 expressing cells (Fig. 5*E*). Based on densitometry of three repeat experiments, D609 reduced PKCα translocation at 1 and 10 min by 76 and 77%, whereas the reduction by U73122 was 27 and 22%, respectively. In contrast, TF-dependent PAR1 activation resulted in transient PKCα translocation that was entirely dependent on PI-PLC. Thus, sustained activation of PKCα and TF cytoplasmic domain phosphorylation are predominantly dependent on a PC-PLC pathway downstream of PAR2 signaling.

DISCUSSION

In this study, we show that PAR2 signaling specifically targets the TF cytoplasmic domain by inducing TF phosphorylation. Our data demonstrate that the ternary TF-VIIa-Xa complex similarly activates PAR1 or PAR2, based on efficiency of PAR internalization and ERK phosphorylation, but only PAR2 signaling leads to TF phosphorylation. TF cytoplasmic domain phosphorylation is dependent on PKCα activation. The presented mutational data support the concept that phosphorylation of the PKC consensus Ser^{253} is necessary for subsequent Ser^{258} phosphorylation, which is recognized by the phosphorylation-specific antibody to TF (22). Rapid PKCα membrane recruitment downstream of PAR1 or PAR2 demonstrates efficient receptor activation by the ternary complex. However, only PAR2 signaling induced sustained PKCα membrane localization, which coincides with the somewhat delayed kinetics of TF phosphorylation. Pharmacological blockade shows that PAR1 and PAR2 activations recruit PKCα through distinct PLC pathways. Sustained activation of PKCα downstream of PAR2 is dependent on PC-PLC, which is known to induce prolonged

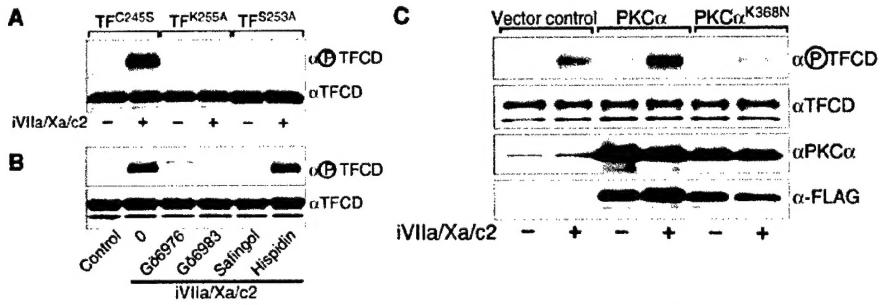

FIG. 4. **Role of PKCα in PAR2-dependent TF cytoplasmic domain phosphorylation.** *A*, HUVECs were co-transduced with C245S, K255A, or S253A mutants of TF and PAR2 with the same virus dose as described for Fig. 2. After 48 h, the cells were transferred to serum-free medium for 2 h and stimulated with ternary complex for 1 h. TF cytoplasmic domain phosphorylation was determined by Western blotting and reprobing with αTFCD. *B*, cells expressing $Cys^{245}Ser$ TF with PAR2 were serum-starved (2 h) and pretreated for 30 min with conventional PKC inhibitors, Gö6976 (1 μM), Gö6983 (1 μM), or isoform specific inhibitors for PKCα, Safingol, (50 μM), and PKCβ, Hispidin, (10 μM), followed by stimulation with ternary complex for 1 h at 37 °C. *C*, cells expressing C245S TF and PAR2 were co-transfected with 2 μg of pcDNA3.1 (vector control), FLAG-tagged PKCα or a catalytically inactive PKCα (K368N) constructs using FuGENE 6. After 48 h, the cells were serum-starved and stimulated with ternary complex for 1 h. *Top panel*, TF cytoplasmic domain phosphorylation by αP-TFCD blot. *Second panel*, TF expression by reprobing with αTFCD. *Bottom two panels*, PKCα expression by anti-PKCα and anti-FLAG blot.

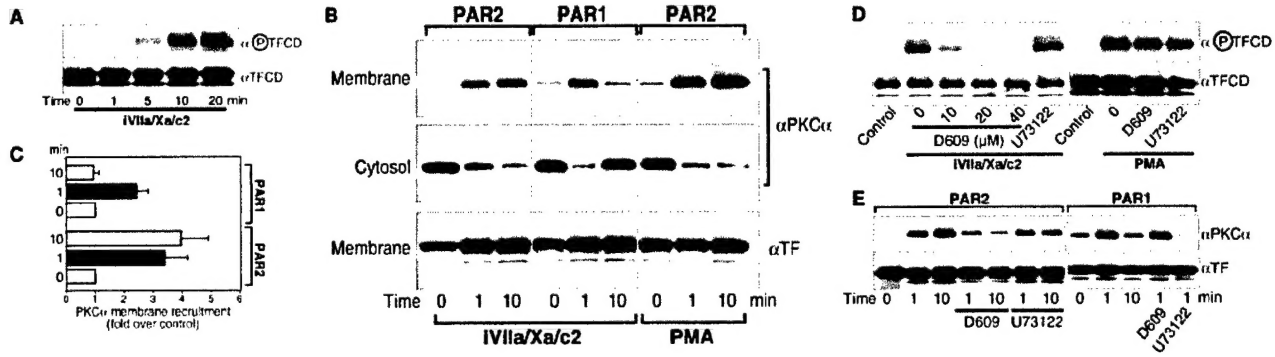

FIG. 5. **PAR2-dependent TF cytoplasmic domain phosphorylation requires PC-PLC-mediated sustained PKCα activation.** *A*, time course of ternary complex-induced TF cytoplasmic domain phosphorylation. HUVECs co-expressing C245S TF and PAR2 were transferred to serum-free medium for 2 h and stimulated with ternary complex for the indicated time. TF cytoplasmic domain phosphorylation was determined by Western blotting and reprobing with αTFCD. *B*, C245S TF and PAR2- or PAR1-expressing cells were serum-starved (4 h) and stimulated with ternary complex for 1 or 10 min followed by preparation of membrane and cytosolic fractions. PKCα distribution was determined by Western blotting of samples with equal protein content. Similar loading of membrane fractions is confirmed by reprobing with α-TF (*bottom panel*). *C*, quantitation of PKCα membrane recruitment by ternary complex signaling in PAR2- or PAR1-expressing cells. Fold inductions of translocation were determined by densitometry (means and standard deviations, $n = 3$). *D*, effect of PC- and PI-PLC-specific inhibitors on TF cytoplasmic domain phosphorylation. HUVECs expressing C245S TF and PAR2 were transferred to serum-free medium for 2 h and preincubated with the indicated concentration of the PC-PLC-specific inhibitor D609 or the PI-PLC inhibitor U73122 (10 μM) for 30 min followed by stimulation with agonist for 1 h. In the *right panel* D609 was used at 20 μM. TF cytoplasmic domain phosphorylation was determined by Western blotting, and equal TF expression was confirmed on separate blots. *E*, HUVECs pretreated with 20 μM D609 or 10 μM U73122 were stimulated, followed by membrane isolation. PKCα membrane recruitment was determined by Western blotting with α-PKCα and reprobing for confirmation of equal protein loading with α-TF. For all panels ternary complex iVIIa/Xa/NAPc2 (10/20/100 nM) and PMA (20 ng/ml) were used.

membrane translocation of PKCs (32). The fact that PMA stimulation, independent of PAR2, induces both TF phosphorylation and sustained PKCα membrane recruitment provides further support for an essential role of prolonged PKCα signaling in TF cytoplasmic domain phosphorylation.

This study adds to emerging evidence (10, 12, 13) that PAR1 and PAR2 signaling elicit specific and only partially overlapping responses in endothelial cells. More importantly, only TF-dependent signaling, but not the signaling by other coagulation factors, induced cytoplasmic domain phosphorylation. The phosphorylation of the TF cytoplasmic domain thus represents a marker for upstream coagulation signaling through PAR2. Both PAR2 and TF are induced by inflammatory mediators in endothelial cells in tissue culture (33, 34), and TF has been detected *in vivo* in splenic endothelial cells in severe systemic inflammation (35) or in breast cancer-associated endothelium (36). Moreover, PAR2 is found to be up-regulated upon differentiation of monocytes into macrophages (37), which also express TF. Thus, PAR2-dependent phosphorylation of TF may play regulatory roles in the biology and pathology of vascular cell types that co-express TF and PAR2.

The role of the TF cytoplasmic domain has previously been analyzed in heterologous transfection systems. Deletion of the entire cytoplasmic domain has little influence on the procoagulant function of TF (27, 38, 39) and on TF-VIIa proteolytic signaling, as measured by immediate early gene induction or phosphorylation events downstream of G protein-coupled receptor signaling (40–42). Although the relevant PARs that transmit TF-dependent signaling in these cell models are incompletely defined, these studies are consistent with an interpretation that the TF cytoplasmic domain is not required for PAR activation. Our data show that TF ternary complex signaling in the case of both PAR1 and PAR2 signaling is rapid, leading to PKCα membrane recruitment within 1 min. However, TF cytoplasmic domain phosphorylation is a delayed response, and it is therefore not surprising that the TF cytoplasmic domain played apparently no role in early responses of TF-VIIa signaling. In addition, TF cytoplasmic domain phosphorylation appears to be more efficient by ternary TF-VIIa-Xa in comparison with TF-VIIa stimulation, at least under our experimental conditions, which may indicate that the TF cytoplasmic domain plays a regulatory role in signaling associated

with activation of the coagulation pathways.

TF is important for metastasis (27, 43), cell adhesion, and migration (44–47). Whereas PAR1 signaling potently induces Rho activation (12, 13), PAR2 only transiently activates Rho while activating Rac and recruiting a β-arrestin scaffolding complex for ERK localization (14, 15). These pathways promote cell motility, a cellular function that is possibly supported by PAR2 signaling. PAR2 signaling that targets the TF cytoplasmic domain may operate in this context, and the presented data provide novel insight into the mechanism by which PAR2 signaling feeds back onto the protease-binding receptor TF that supports PAR cleavage by associated proteases. Phosphorylation of the TF cytoplasmic domain may influence the recruitment of adaptors and thus integrate and regulate nonhemostatic roles of TF in cell migration, angiogenesis, and inflammation.

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